

METHOD FOR ASSESSING PIGMENTED SKIN

FIELD OF INVENTION

The present invention relates to the assessment of
5 pigmented skin.

BACKGROUND OF THE INVENTION

The chromatic characteristics of skin color arise
from the interactions of light (primarily absorption and
10 scattering) with the epidermis and the dermis. The
primary light absorbers in skin are hemoglobin and
melanin. Most of scattering is attributed to collagen
fibers and in pigmented skin to melanosomes.
Traditionally, skin redness is considered to arise due to
15 locally elevated concentrations of hemoglobin, whereas
skin pigmentation is attributed to melanin.

Human skin is structurally and optically
heterogeneous. It consists of two discrete layers, the
epidermis and the dermis, each with different biological
20 structure and different optical properties arising from
their individual absorbing and scattering constituents.
The epidermis is a highly cellular tissue consisting of
layers of keratinocytes. It is known that the primary
absorber in the epidermis is melanin. It has also been
25 shown (Kollias, et al., (1991) J Photochem Photobiol B
9(2): 135-160) that particulate melanin is a major
contributor to epidermal scattering. Melanin is
synthesized in specialized organelles, the melanosomes,
which are manufactured in the melanocytes that are found
30 at the basal layer of the epidermis. The melanosomes are
secreted from the dendritic processes of the melanocytes,
and they are phagocytosed by the keratinocytes.

The dermis is essentially an acellular tissue
sparsely populated by fibroblasts. It consists primarily

of extracellular matrix components (collagen and elastin), blood vessels, and lymphatic vessels. Collagen microfibrils are organized into fibers and the fibers into bundles. These structures constitute the major cause of light scattering in the skin. Hemoglobin is found in the dermal blood supply and is responsible for the red appearance of skin, as in the case of erythema. Skin hemoglobin is confined in networks of arterial and venous plexi that run approximately parallel to the skin surface and in small capillaries that run vertical to the skin surface and reach close to the dermal-epidermal junction in the form of capillary loops. Anatomically, one can distinguish a superficial arterial and venous plexus and a deeper plexus. The capillaries stem from the superficial arterial plexus and empty in the superficial venous plexus. The superficial and deeper plexi are interconnected through smaller vessels. At the deeper level, arteriovenous anastomoses (shunts) provide ways for blood flow to bypass superficial skin layers, thus enabling skin thermal regulation. Circulating erythrocytes in the blood vessels contain high concentrations of hemoglobin. The hemoglobin molecule has four heme groups, which can bind to and deliver oxygen molecules to the tissues. When these binding sites are unoccupied, the molecule is called deoxy-hemoglobin (deoxy-Hb) or reduced hemoglobin. When oxygen molecules occupy these binding sites, it is termed oxy-hemoglobin (oxy-Hb) or oxygenated hemoglobin. Each form of hemoglobin has its own characteristic absorption profile (Kollias, (1995) Clin Dermatol 13(4):361-367).

It follows from the above that the visual perception of skin color is the cumulative result of contributions of

various optically active molecules that are found in
varying concentrations in the skin. The relative
contributions of each chromophore can be evaluated
quantitatively by analyzing the remittance spectra of skin
tissue. Applicants have discovered that deoxy-hemoglobin
not only contributes to erythema, but also surprisingly
contributes to perceived pigmentation of the skin.
Applicants have accordingly modified the analysis of
remittance spectra to take into account this discovery.

SUMMARY OF THE INVENTION

In one aspect, the present invention features a
method of approximating the relative contribution of
melanin responsible for the perceived pigmentation of an
area of skin including the steps of (i) determining the
absorbance of light at a wavelength of from about 620 nm
to about 750 nm at such area of skin and (ii) subtracting
the approximate relative contribution of deoxy-hemoglobin
from such absorbance.

In another aspect, the present invention features A
method of approximating the relative contribution of
deoxy-hemoglobin responsible for the perceived
pigmentation of an area of skin including the steps of (i)
determining the absorbance of light at a wavelength of
from about 550 nm to about 590 nm at such area of skin and
(ii) subtracting the approximate relative contribution of
oxy-hemoglobin and melanin from such absorbance.

In another aspect, the present invention features a
method of approximating the relative amount of melanin
responsible for the perceived pigmentation of an area of
skin, including the steps of: (i) measuring the
reflectance of a first light at a wavelength of from about

555 nm to about 565 nm, a second light at a wavelength of
from about 570 nm to about 585 nm, a third light at a
wavelength of from about 620 nm to about 650 nm, and a
fourth light at a wavelength of from about 680 nm to about
750 nm at such area of skin; (ii) determining the first
approximate relative contribution of melanin to such
perceived pigmentation by determining the absorbance of
the third light and the fourth light at such area of skin;
(iii) subtracting the first approximate relative
contribution of melanin from the determined absorbance of
the first light and the second light at such area of skin;
(iv) determining the first approximate relative
contribution of deoxy-hemoglobin from the recalculated
absorbance of the first light and the second light of step
(iii); and (v) subtracting the first approximate relative
contribution of deoxy-hemoglobin from the first
approximate relative contribution of melanin to obtain a
final approximate relative contribution of melanin.

In another aspect, the present invention features a
method of approximating the relative amount of deoxy-
hemoglobin responsible for the perceived pigmentation of
an area of skin including the steps of: (i) measuring the
reflectance of a first light at a wavelength of from about
555 nm to about 565 nm, a second light at a wavelength of
from about 570 nm to about 585 nm, a third light at a
wavelength of from about 620 nm to about 650 nm, and a
fourth light at a wavelength of from about 680 nm to about
750 nm at such area of skin; (ii) determining the first
approximate relative contribution of melanin to such
perceived pigmentation by determining the absorbance of
the third light and the fourth light at such area of skin;
(iii) subtracting the first approximate relative

contribution of melanin from the determined absorbance of the first light and the second light at such area of skin; and (iv) determining the first approximate relative contribution of deoxy-hemoglobin from the recalculated absorbance of the first light and the second light of step (iii).

In another aspect, the present invention features a method of approximating the relative contribution of melanin to a perceived pigmentation of an area of skin including the steps of: (i) examining such skin with a device including a light source and a reflectance detector, wherein the detector measures the reflectance from such area of skin of light generated by the light source of at a first wavelength of from about 555 nm to about 565 nm, at a second light wavelength of from about 570 nm to about 585 nm, at a third wavelength of from about 620 nm to about 650 nm, and at a fourth wavelength of from about 680 nm to about 750 nm at such area of skin; (ii) determining the first approximate relative contribution of melanin, to such perceived pigmentation by using the calculated absorbance of the third wavelength and the fourth wavelength at such area of skin; and (iii) further determining the approximate relative contribution of melanin by subtracting from the first approximate relative contribution of melanin the approximate relative contribution of deoxy-hemoglobin determined by the absorbance of the first wavelength and the second wavelength at such area of skin.

In another aspect, the present invention features a method of approximating the relative contribution of deoxy-hemoglobin to a perceived pigmentation of an area of skin including the steps of: (i) examining such area of

skin with a device including a light source and a reflectance detector, wherein the detector measures the reflectance from such area of skin of light generated by the light source of at a first wavelength of from about 555 nm to about 565 nm, at a second light wavelength of from about 570 nm to about 585 nm, at a third wavelength of from about 620 nm to about 650 nm, and at a fourth wavelength of from about 680 nm to about 750 nm at such area of skin; (ii) determining the approximate relative contribution of melanin to such perceived pigmentation by using the calculated absorbance of the third wavelength and the fourth wavelength at such area of skin; and (iii) determining the approximate relative contribution of deoxy-hemoglobin by subtracting the first approximate relative contribution of melanin from the calculated absorbance value at the first wavelength and the second wavelength.

Other features and advantages of the present invention will be apparent from the detailed description of the invention and from the claims.

BRIEF DESCRIPTION OF FIGURES

Fig 1a is a graph showing the change in concentration of oxy-hemoglobin at various SSR dosages.

Fig 1b is a graph showing the change in concentration of deoxy-hemoglobin at various SSR dosages.

Fig 1c is a graph showing the change in concentration of melanin at various SSR dosages.

Fig 2 is a graph showing the change in concentration of oxy-hemoglobin, deoxy-hemoglobin, melanin, and scattering at various applied pressures.]

Fig 3 is a graph showing that perception of "skin pigmentation" depends on deoxy-hemoglobin concentration in a similar fashion to its dependence on melanin.

Fig 4 is graph showing the change in concentration of oxy-hemoglobin, deoxy-hemoglobin, melanin, and scattering following topical application of 3% H₂O₂

DETAILED DESCRIPTION OF THE INVENTION

It is believed that one skilled in the art can, based upon the description herein, utilize the present invention to its fullest extent. The following specific embodiments are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Also, all publications, patent applications, patents, and other references mentioned herein are incorporated by reference.

The present invention relates to a method of approximating the relative contribution of melanin and/or deoxy-hemoglobin responsible for the perceived pigmentation of an area of skin. What is meant by the term "relative contribution" is either the (i) the concentration of the chromophore (e.g., melanin or deoxy-hemoglobin) at such area of skin or (ii) the percentage of the perceived pigmentation that is a result of the presence of such chromophore at the area of skin. Examples of pigmented areas of the skin include, but are not limited to, freckles, age spots, hyperpigmentation, dark circles, or tanned skin). The methods of the present

invention utilize the absorbance of light at various wavelengths. What is meant by the phrase "a light at a wavelength" is radiation band centered at the specified wavelength wherein such band has a full width at half-maximum intensity of less than about 10 nm (e.g., for example less than about 5 nm).

The methods and devices of the present invention can be used to determine the source(s) of perceived pigmentation on areas of the skin. For example, if it is determined that the melanin contributes to the perceived pigmentation, skin lightening/depigmenting agents can be used to treat the area. Examples of such agents include, but are not limited to, ascorbic acid, kojic acid, and soy extracts. If it is determined that the deoxy-hemoglobin contributes to the perceived pigmentation, vasoactive agents can be used to treat the area. Examples of such agents include cortisone and hydrogen peroxide.

Objective quantitative evaluation of skin color using non-invasive instrumentation has been used since the early decades of the 20th century (Brunsting, L. A. and C. Sheard, (1929), J Clin Invest 7(4): 575-592). Since then spectrophotometers have become smaller and simpler in use.

Many researchers have used methods based on reflectance measurements that either give "erythema" and "pigmentation" indices based on simple calculations (Diffey, et al., (1984) Br J Dermatol 111:663-672) or calculate tri-stimulus values ($L^*a^*b^*$ scale) that have been adopted by the international committee of standards (CIE) as the preferred method for color measurement (Westerhof, et al., (1986) Photodermatol, 3:310-314). In the latter method, L^* and b^* , as well as combinations of the two, have been used as "pigmentation" parameters, and

a* as the "erythema" parameter. Recent studies have showed that in both methods what is clinically perceived erythema and pigmentation does not correlate linearly with the calculated indices (Takiwaki, et al., (2002), Skin Res Techn 8:78-83 and Wagner et al., (2002), Pigment Cell Res, 15:5:379-384). A more accurate method is to analyze the remitted spectrum to its constituents based on Diffuse Reflectance Spectroscopy (DRS) (Kollias, et al., Clin. Dermatol. 13:36 (1995)). Apparent concentrations of melanin, oxy-Hb, and deoxy-Hb can be extracted from absorption spectra obtained by DRS, thus separating the vascular from the melanin reactions that are responsible for erythema and pigment. DRS measurements are rapid, non-invasive, objective, and quantitative. Instruments that perform DRS can be small, portable, and easy to use.

The absorbance curve was calculated as the logarithm of the ratio of the diffuse reflectance from a non-irradiated site to the diffuse reflectance from an irradiated site. In previous algorithms, pigment was evaluated from the absorbance curve as the slope of the fitted straight line over the wavelength range of 620-720 nm. Then, the absorbance curve was corrected for the pigment absorption and, finally, the oxy-Hb and deoxy-Hb absorption curves were fitted in the range of 550-580 nm, where they exhibit maxima. Such algorithms, however, over-estimate the contribution of melanin and under-estimate the contribution of hemoglobin to the perceived pigmentation.

It has been assumed that deoxy-hemoglobin contributes to the "red" color in skin just like oxy-hemoglobin, afterall, both of these chromophores appear red in vitro. Deoxy-hemoglobin in skin is found in the superficial

venous plexus and in particular in the venules. These vessels are large enough (20-50 microns in diameter) so that light (in the yellow-green part of the spectrum) is almost completely attenuated when it tries to traverse through them. This means that they appear dark (almost black) and slightly reddish. Slight occlusion of blood flow or compression of venules causes an increase or a decrease in the amount of deoxy-hemoglobin resident in the venules and therefore a change in the color of the skin. A surprising result was that these changes in concentration of deoxy-hemoglobin in the skin appear like changes in melanin pigment.

The main reason why deoxy-hemoglobin contributes to a pigmented skin appearance is that the absorption spectrum of deoxy-hemoglobin in the 630-700 nm range is very similar to the absorption spectrum of epidermal melanin, so whatever light is transmitted has the color balance of pigment. Furthermore, the size of the vessels in the superficial venous plexus is such that the transmitted radiation through these vessels is approximately 50% lower than the incident intensity, and, therefore, they appear dark. Moreover, deoxy-hemoglobin is a dominant pigment in normal skin, contributing 30-50% to the perceived concentration of blood and equal in its contribution to that of oxy-hemoglobin and melanin to skin color.

Device for Reflectance Acquisition

In one embodiment, the reflectance acquisition can be accomplished by either point spectroscopy or multi-spectral imaging. In the case of point spectroscopy, the system can, in one embodiment, include a light source, a probe, and a reflectance detector. In one embodiment, the

light source is a broad-band source that includes wavelengths from about 560 to about 780 nm. Examples of such broadband sources include, but are not limited to, incandescent light sources, metal-halide light sources, halogen lamps, and broad-band light emitting diodes (LEDs). In one embodiment, the light source is a monochromatic or narrow band source (e.g., emitting a wavelength or wavelengths of light substantially within the range of from about 560 to about 780 nm). Examples of such light sources include, but are not limited to, tunable lasers, narrow-band LEDs, and filtered broad-band sources. A combination of two or more of the above light sources can also be used. Examples of the probes include, but are not limited to, a fiber optic bundle (e.g., for contact with the skin site of interest) or an integrating sphere (e.g., with an opening that comes in contact with the site of interest). In the case of an integrating sphere, care should be taken to account for the contribution of specular reflection to the total reflected signal. In the case where a broad-band light source is used, a dispersive element (such as a grating or a monochromator), or a filter (such as a narrow band interference filter or a liquid crystal tunable filter) should be used to filter the light prior to entering the detector (e.g., to filter the light prior to contact with the skin or to filter the reflected light off the skin prior to entering the detector). Examples of detectors include, but are not limited to, a single photodiode, a photodiode array, a CCD array, or a photomultiplier.

In the case of multispectral imaging (e.g., a series of images acquired at a selected few wavelengths of interest), the system can include a light source and an

imaging detector. Examples of light sources include, but are not limited to, broadband or narrow band sources capable of illuminating the area of interest or a combination of light sources as discussed above. The
5 detector can be a digital camera, such as a CCD or CMOS. In the case of broad-band light source, the image needs to be filtered before reaching the detector to the appropriate wavelengths. In one embodiment, filtering can be accomplished by placing appropriate dispersive element
10 or filter between the light source and the detector to filter the light entering the detector. In one embodiment for multispectral imaging, orthogonal polarization between the illumination and the detector is used in order to eliminate specular reflection (e.g., glare). Orthogonal
15 polarization can be accomplished by placing a linear polarizer in front of the light source and a second linear polarizer in front of the detector (e.g., the camera). The second linear polarizer can be placed in such a way that its polarization plane is orthogonal to the
20 polarization plane of the first linear polarizer.

Algorithm for Analysis of Absorbance

Applicants have discovered a new corrected algorithm for the analysis of reflectance spectra from skin that
25 separates the approximate vascular from the approximate melanin contributions to the perceived erythema and/or pigmentation by taking into account the spectral contribution of deoxy-Hb in the red region of the spectrum (620-700 nm). In one embodiment, the procedure is as
30 follows: (a) the diffuse reflectance spectrum from skin is referenced to uninvolved normal skin of the same individual, (b) the spectrum is fitted with a straight

line in wavelength ($f(\lambda)$) in the spectral range 620-720 nm (the slope of the straight line is believed to be related to the concentration of melanin in the skin); (c) the straight line that best fits the data is then subtracted from the whole spectrum, which results in a spectrum that is identical with the baseline at wavelengths in the range 620-720 nm and deviating from baseline to shorter wavelengths; (d) the difference spectrum is fitted using the absorption parameters of oxy-hemoglobin and deoxy-hemoglobin at the wavelengths of 560 and 578 nm, which is accomplished by solving a system of two equations and two unknowns, the result is an apparent concentration of oxy-hemoglobin and of deoxy-hemoglobin; (e) the concentration of deoxy-hemoglobin is used to calculate the contribution of this chromophore to the measured slope of the fitted line of step (b); (f) the apparent concentration of melanin is related to the slope of the line of step (b) corrected for the absorption of deoxy-hemoglobin in this wavelength range given by step (e); (g) an apparent scattering parameter can be calculated from the value of the corrected fitted line of step (f) at an arbitrary selected wavelength in the range of 630 - 820 nm; (h) the corrected fitted line of step (f) is subtracted from the whole spectrum (similarly to step (c)), and (i) the new difference spectrum is fitted using the absorption parameters of oxy-hemoglobin and deoxy-hemoglobin at the wavelengths of 560 and 578 nm (similarly to step (d)), which is accomplished by solving a system of two equations and two unknowns, the result is the corrected apparent concentration of oxy- and of deoxy-hemoglobin.

In one embodiment of the algorithm, the above steps can be condensed to the following equations:

The intercept (int_o) and the slope (m_o) of the absorbance spectra in the 620 - 720 nm range can be calculated from the values of the spectrum at the wavelengths 620 nm and 720 nm (S^{620} and S^{720} correspondingly):

$$int_o = \frac{720 \times S^{620} - 620 \times S^{720}}{720 - 620}, \quad m_o = \frac{S^{720} - S^{620}}{720 - 620} \quad (1)$$

The absorbance values at 560 nm and 578 nm corrected for melanin (initial approximation) are given by:

$$S_c^{560} = S^{560} - (m_o \times 560 + int_o), \quad S_c^{578} = S^{578} - (m_o \times 578 + int_o) \quad (2)$$

The concentrations of oxy-Hb ($[HbO_2]$) and deoxy-Hb ($[Hb]$) can be calculated from these corrected values of the absorption spectrum and the extinction coefficients of oxy-Hb and deoxy-Hb at 560 nm and 578 nm, $\alpha_{HbO_2}^{560}$, $\alpha_{HbO_2}^{578}$, α_{Hb}^{560} , and α_{Hb}^{578} :

$$[HbO_2] = \frac{\alpha_{Hb}^{560} \times S_c^{578} - \alpha_{Hb}^{578} \times S_c^{560}}{\alpha_{Hb}^{560} \times \alpha_{HbO_2}^{578} - \alpha_{Hb}^{578} \times \alpha_{HbO_2}^{560}}, \quad [Hb] = \frac{\alpha_{HbO_2}^{578} \times S_c^{560} - \alpha_{HbO_2}^{560} \times S_c^{578}}{\alpha_{Hb}^{560} \times \alpha_{HbO_2}^{578} - \alpha_{Hb}^{578} \times \alpha_{HbO_2}^{560}} \quad (3)$$

The approximate relative contribution of oxy-hemoglobin and deoxy-hemoglobin can be used to examine vascular components of the skin and its related skin conditions, such a erythema, acne, inflammation, rosacea, and spider veins.

Finally, the corrected melanin concentration can be calculated given the slope of the deoxy-Hb extinction coefficient in the range 620-720 nm, $SI_{Hb}^{620-720}$:

$$[melanin] = m_o - [Hb] \times SI_{Hb}^{620-720} \quad (4)$$

The corrected values for oxy-Hb and deoxy-Hb concentrations can be calculated from equations (2) and (3) after substituting m_o with the corrected melanin concentration given in equation (4).

The approximate relative contribution of the light scattering can be calculated from:

$$sc = m_o \times \lambda_{sc} + int_o \quad (5)$$

where λ_{sc} is the chosen wavelength in the range 630-820nm. The approximate relative contribution of scattering can be used to examine dermal collagen and its related skin conditions, such as wrinkles and fine lines.

The above algorithm can be applied to point spectroscopy as well as to hyper- or multi-spectral imaging. Hyper-spectral imaging refers to a series of images acquired at different wavelengths, in such a way that a spectrum can be reconstructed for each individual pixel of the image based on the intensity values of the particular pixel throughout the wavelength range of acquisition. Multi-spectral imaging refers to a series of images acquired at a selected few wavelengths of interest.

Before any analysis of the hyper- or multi-spectral images takes place, care should be taken that the spectral images are registered to cancel any motion artifacts that may have occurred during image acquisition. In one embodiment, image registration includes image translation

and rotation, but not image dilation, as the latter may compromise the quantitative nature of the algorithm.

In the case of hyper-spectral imaging, the plot of the intensity value of a pixel versus the acquisition wavelength is equivalent to a reflectance spectrum (scaled from 0 to 255 for 8 bit images) of the imaged object at the spatial position of the pixel. The following procedure describes, in one embodiment, how to convert the hyper-spectral spectrum of a pixel to an absorbance spectrum: (a) the percent reflectance spectrum is calculated by taking the ratio of the pixel intensity values to either the maximum allowed intensity (255 for 8 bit images), the reflectance from a white standard, or an image from a wavelength from about 800 to about 900 nm; (b) the estimated specular reflectance is subtracted from the ratio of step (a) (specular reflectance is given by the Fresnel law and for human skin this value is approximately 0.04); and (c) the negative logarithm of the corrected ratio of step (b) plotted against the acquisition wavelengths is the resulting absorbance spectrum.

Once the absorbance spectrum has been calculated, the procedure described above can be used to evaluate the chromophore values at each pixel. After normalization (byte scaling), the values of each chromophore for all pixels can be displayed as separate images, also referred to as "chromophore maps." See, e.g., Stamatas et al., (2003) Proc. SPIE 4959:77-82 for chromophore maps calculated by a previous algorithm.

In one embodiment, a similar "curve-fitting" procedure can be used to calculate apparent values of other chromophores native to human skin or externally

applied. Some examples of native pigments not mentioned above include, but are not limited to, met-hemoglobin (absorbance at 404 nm and 635 nm), bilirubin (absorbance at 460 nm), beta-carotene, and water (absorbance bands at 970 nm, 1100 nm, etc.). Externally applied chromophores include, but are not limited to, materials that absorb in UV, visible, or NIR, such as sunscreens, cosmetic formulations, make-up, lipstick, and topical drugs. Thus, a similar approach can be used to quantify deposition of an ingredient of a topical formulation.

Alternatively to hyper-spectral imaging a small number of acquisition wavelengths can be selected that would result in chromophore maps using the above procedure. This approach is termed multi-spectral imaging. The requirements for selecting the right acquisition wavelengths are as follows: (a) One image is required to be acquired in the wavelength region of the deoxy-hemoglobin maximum at 560 nm and has to have a bandwidth of less than 5 nm at each side, in order to avoid the maxima of oxy-hemoglobin at 555 nm and 578 nm; (b) One image is required to be acquired in the wavelength region of the beta band of oxy-hemoglobin (at 578 nm) and has to have a bandwidth of less than 10 nm at each side; and (c) At least two images are required to be acquired in the wavelength region of 620 nm - 720 nm with the wavelengths being chosen as far from each other as possible to cover evenly the region.

Melanin and scattering values can be calculated from the (c) images as described in the procedure above and oxy- and deoxy-hemoglobin values from a 2x2 system using the intensities of images (a) and (b). To calculate

values for the four chromophores mentioned here, a minimum of four images at different wavelengths should be used.

Clinical Studies

5 Twelve healthy individuals with skin photo-types III-IV participated in the study. The source of irradiation was a 500W UVC-filtered Xenon arc solar simulator (Solar Light Co., Philadelphia, PA). The instrument was calibrated right before its use, and the total power of
10 the source was recorded every 3 to 4 hours throughout the day to assure its stability and spectral quality following Colipa (The European Cosmetic, Toiletry, and Perfumery Association) guidelines. Initially the minimum solar simulator radiation (SSR) dose to induce perceptible
15 erythema (MED) was determined on the back of each participant. Clinical erythema was evaluated 24 hours after the irradiation. Following MED determination, each individual was irradiated on the back with SSR doses of 0.7, 1.0, 1.5, 2.1, and 3 MED. The skin reactions were
20 evaluated on days 1, 7, 14, and 21 after exposure. Evaluations included cross-polarized photography, DRS measurements, and clinical assessment of erythema and pigmentation by an experienced dermatologist.

 In a second experiment, a pressure cuff was applied
25 to the upper arm of ten healthy volunteers. The applied pressure was set at levels of 0, 20, 30, 40, and 60 mmHg.

 Measurements were taken 5 min after each pressure level was set to allow for the vasculature to equilibrate. Changes in skin color due to vascular reactions were
30 evaluated visually with a chromameter and with a DRS instrument.

In a third experiment, 2 inch diameter cotton pads soaked in 3% H_2O_2 (U.S.P. topical anti-infective) in aqueous solution were applied on the volar forearm of ten healthy volunteers for 1 min. The pads were removed and the skin area was dried with fresh cotton pads. DRS measurements and visible evaluation of the treated sites were performed at 0, 5, 10, 15, and 20 min after removal of the pads.

Diffuse Reflectance Spectroscopy (DRS)

The DRS instrument contained a quartz halogen light source (Ocean Optics, Boca Raton, FL), a bifurcated fiber bundle (Multimode Fiber Optics, East Hanover, NJ), an S2000 spectrometer (Ocean Optics, Boca Raton, FL), and a laptop computer (Toshiba Tecra, Irvine, CA). One leg of the fiber bundle was connected to the light source and the other to the spectrometer. Measurements were performed by placing the common end of the fiber bundle gently in contact with skin so as not to perturb the blood content.

A reflectance spectrum was acquired in the range of 400-820 nm. Apparent concentrations of hemoglobin and melanin were calculated from the diffuse reflectance spectra. See, e.g., Kollias et al., *Photodermatol* 5:53-60 (1988). Briefly, the absorbance curve was calculated as the logarithm of the ratio of the diffuse reflectance from a non-irradiated site to the diffuse reflectance from an irradiated site. Pigment was evaluated from the absorbance curve as the slope of the fitted straight line over the wavelength range of 620-720 nm. Then, the curve was corrected for the pigment absorption, and finally, the oxy-Hb and deoxy-Hb absorption curves were fitted in the

range of 550-580 nm, where they exhibit maxima, as set forth in Table 1.

Table I

Chromophore	Absorption curve characteristic
Melanin	Monotonic increase towards short wavelengths; Approximates linear in the region 600 - 750 nm
Oxy-Hemoglobin	Maxima at 415, 540, and 577 nm
Deoxy-Hemoglobin	Maxima at 430 and 555 nm

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The reproducibility of the method for calculating apparent hemoglobin concentrations was calculated as the error between measurements, and it was found to be better than 10%. It needs to be noted that for the collection geometry used here, an under-estimation of the reflectance at the long wavelengths (red/NIR region) compared to the shorter wavelengths (blue/green region) is anticipated. However, the measurements were always performed relative to baseline, or to neighboring untreated skin, and, therefore, such artifacts have been normalized.

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Data Analysis

Linear regressions of the data were calculated using the least square errors algorithm. The goodness of fit is given by the correlation coefficient (R-squared).

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Statistical significance was calculated using the Student's t-test for paired data distributions.

UV Irradiation Experiment

5 UV-induced erythema was typically evaluated on day 1 after irradiation, while pigmentation was evaluated on day 7. The time course of changes in the concentration of oxy-Hb, deoxy-Hb, and melanin are shown in Figs 1a, 1b, and 1c. On day 1, after irradiation the observed skin
10 reaction was classified as clinical "erythema". The visual observation of erythema correlated well with a dramatic increase in oxy-Hb and deoxy-Hb, as calculated from the spectroscopic data (Figs. 1a and 1b). The observed skin reaction on day 7 was classified clinically
15 as "pigmentation", which correlated with an increase in melanin (Fig. 1c). However, the levels of both hemoglobins were significant on day 7, indicating that there is a strong vascular contribution to the observed reaction (Figs. 1a and 1b). On day 14, the reaction was
20 again classified as "pigmentation", and although the melanin levels remained elevated, the concentration of deoxy-Hb was still above baseline (Fig. 1b). On day 21, the observed pigmentation was almost exclusively due to melanin (Fig. 1c). Although deoxy-Hb is still measurably
25 above baseline, its contribution at 0.1 level was not visibly perceptible.

Pressure cuff experiment

30 In the pressure cuff experiment, increasing pressure resulted in a reduction of the values of L^* and b^* as measured by the chromameter corresponding to darker and less yellow color respectively. On the contrary, the

value of a^* increased corresponding to a more red appearance. Although the color changes were recorded with the chromameter, it is of interest to note that visually the change in skin color was hard to observe. The reason was that the human eye works better in contrast and since the pressure was applied on the whole of the arm, the color change was uniform and, therefore, difficult to detect. DRS analysis showed that the only chromophore that was affected by changing the applied pressure was deoxy-Hb, which increased linearly with pressure (Fig. 2).

Oxy-Hb and melanin remained practically unaltered. The characteristic angle, $\alpha = \arctan ((L^*-50)/b^*)$, is a measure of the perceived pigmentation in such a way that when apparent pigmentation increases this parameter decreases. See, e.g., Park, et al., Clinexp Dermatol 24:315-320 (1999). In the present experiment, the characteristic angle decreased with increasing pressure in all volunteers, indicating that pressure-induced increases in blood stasis can be perceived as increased pigmentation (Fig. 3).

Hydrogen Peroxide Experiment

Application of cotton pads soaked in 3% hydrogen peroxide for 1 min induced a decrease in perceptible skin pigmentation that lasted for 10-15 min after removal of the pads. Analysis of DRS spectra showed that deoxy-Hb significantly decreased during the period of blanching (Fig. 4). Oxy-Hb decreased slightly, but within instrument variability (determined to be ± 0.1 oxy-Hb units). Melanin and dermal scattering remained unchanged at baseline levels.

It is understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate, and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the claims.

What is claimed is: